Productive and non-productive phases during long-term persistence of influenza C virus

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Persistent infection with a variant of influenza C/Ann Arbor/1/50 virus in MDCK cells has been previously reported. However, the precise molecular mechanism of persistence is still unknown. We show that the release of active progeny virus, as tested for by haemagglutination and acetylesterase profiles, does not take place in freshly seeded MDCK cells. Productive virus replication occurs simultaneously with massive production of structural proteins as shown by immunoprecipitation and immuno-fluorescence. PCR for the HEF structural protein-encoding segment 4 revealed that positive-sense RNA is present only during virus multiplication whereas negative-sense RNA appears to be constantly detectable. In this study we give initial evidence that influenza C virus can persist in the form of its genomic minus strand RNA, and plus strand transcription, protein synthesis and virus replication remain restricted to productive phases.

Influenza C virus, an enveloped and segmented RNA virus of negative single-strand organization, possesses biological properties which are distinct from pathogenic influenza A and B viruses (Smith & Palese, 1989). Its association with mild and mostly symptomless infections in humans is reflected in the lack of influenza-like, respiratory cytopathology. The non-lytic strategy of infection predisposes viruses to generate genetic variants, suitable for persistent maintenance in their natural host (Oldstone, 1991). Considering this, influenza C virus is a good prospective candidate amongst the orthomyxovirus group to propagate spontaneous variants with the capability to undergo long-term persistence in vivo. In cell culture, persistent infection with influenza A virus, as a consequence of evolution of virus mutants, has been investigated for a long time (Frielle et al., 1984). Recent reports underlined the possibility of host cell-mediated selection of influenza C virus also (Umetsu et al., 1992). Madin-Darby canine kidney (MDCK) cells were shown to support well established persistence of influenza C virus (Goshima & Maeno, 1989). Initial characterization of cellular and viral parameters of persistence have been published (Camilleri & Maassab, 1988). In this communication we attempt to verify some of the regulatory mechanisms.

An MDCK cell line persistently infected with influenza C/Ann Arbor/1/50 virus has been stably passaged for more than 5 years (Camilleri & Maassab, 1988). The cells show typical morphological effects, e.g. intracytoplasmic inclusions, but the general growth behaviour is unaffected. The test monolayers grew to confluence within 2 days and continued their cell division at a steady state at a total cell number of approximately 10^7 per culture. By monitoring the virus production and release in this culture, we were able to recognize distinct phases (Fig. 1). By the measurement of the haemagglutination (HA) titre and the acetylesterase activity of the HEF structural protein (Herrler & Klenk, 1991), a non-productive period was detected during the first week after seeding. Thereafter, rapid increase of both activities occurred regularly in separate experiments, but virus expression was fluctuating.

This observation was confirmed by protein analysis, using influenza C virus-specific antisera and monoclonal antibodies. In vivo labelling of proteins was carried out in culture at different times after seeding (Fig. 2). Immunoprecipitation of cellular extracts revealed marked differences. Although all structural proteins examined, i.e. HEF, NP (nucleoprotein), and M, were reliably synthesized in late phases of cell culture, no such signals were obtained from an early, non-productive state. (Their synthesis was undetectable, even when the films were overexposed.)

These data were confirmed by parallel immunofluorescence tests (Fig. 3). In HA-productive cell preparations, the viral antigens HEF, NP and M were clearly
proven and identified by their appropriate localization in the cytoplasmic membrane, nucleus or nucleus/cyttoplasm, respectively. NP protein appeared to be synthesized in abundant quantities. Again, cell preparations from non-productive phases failed to give any positive signal. (Uninfected cell controls were performed routinely.)

We then investigated whether viral RNA, i.e. minus strand (virion RNA) or plus strand (cRNA and mRNA), remained detectable in the presence and absence of viral proteins. For this, we applied a strand-specific amplification test for the HEF-encoding segment 4. Primers from nucleotide positions 22 (5' ATGTTTTTCTCA-TTACTCTTGATGTGGCC 3') to 52 and 1024 (3' CCGTCTTTAGACTGGTACGTACC 5') to 1048 were applied in separate reverse transcription (RT) reactions in order to distinguish between positive- and negative-sense templates. The first PCR was accomplished with both primers. Nested primers for the second round of PCR were taken from nucleotide positions 510 (5'ACATTTGACTGACCTTG 3') to 526 and 988 (3' CCTGATCAGTGACGGTATTAGT 5') to 1012. (All positions indicated in the HEF sequence are numbered according to its coding-sense orientation, as published earlier; Buonagurio et al., 1985.)

RNA from persistently infected cells was prepared by standard procedures (Chomczynski & Sacchi, 1987) at the time points indicated (Fig. 4). All cells used were close to or beneath the detection limit of virus production, as tested by HA from supernatant samples (i.e. Fig. 4 lanes 1 to 4 represent HA titres < 1:1, < 1:1, 1:1 and 1:2, respectively). The first round of PCR did not amplify the expected 1027 bp product to detectable amounts in any case. One-tenth of the final assay volume was subjected to a second round of PCR, using nested primers, and by these means the calculated 503 bp product was amplified to levels detectable by PAGE. (A negative control from uninfected cells proved the absence of contaminants.) Minus strand RNA was amplified in all samples, irrespective of the state of virus activity. Plus strand RNA remained undetectable in samples from the non-productive phase; however, positive signals arose in those cases where at least low virus production was determined in parallel. The detection limit of our RT/PCR conditions was determined by serial dilution of virion RNA, prepared from egg-grown influenza C/Ann Arbor/1/50 virus. One-hundred pg of RNA was found to set the cut-off point for non-radioactive nested PCR experiments, corresponding to 1.43 × 107 virus particles or haploid genomic segments, respectively (results not shown).

The objective of this series of experiments is to clarify the state of expression during persistent influenza C virus infection and the nature of viral RNA and protein continuation. Our data emphasize the periodic occurrence of non-productive phases. Freshly seeded cells, active in metabolism and cell division, fail to produce detectable virus yields. Protein synthesis is reduced to very low levels and minus strand RNA is the only viral product detectable so far. Confluent cell layers may also periodically undergo changes of virus expression with non-productive phases. It is possible that this behaviour reflects cellular alterations and selection of more or less susceptible carrier subpopulations in culture. A mode of changing permissiveness is in line with reports from a reovirus system, where cell modifications were shown to be due to virus persistence (Montgomery et al., 1991).
Fig. 2. Immunoprecipitation of viral proteins from MDCK cells, in the non-productive (a) and productive phase (b) of persistent infection, i.e. 4 days and 16 days after seeding, respectively. Detailed procedures were described previously (Marschall et al., 1989). In brief, cell culture proteins were labelled in vivo by incubation of 25 µCi of [35S]methionine/cysteine (ICN Radiochemicals) overnight. In order to prevent non-specific reactions the extracts were pretreated with plain Protein A and all antibodies were preadsorbed with uninfected cell lysates. A polyspecific rabbit serum (H1/66), raised against influenza C/Johannesburg/1/66 virions, and monoclonal antibodies (MAbs) directed against HEF (FCDD4.1/DA2.6.4), NP (F17/H12), M protein (L2/G32) and an irrelevant control antibody were incubated with the protein extract, representing 10⁶ cells in each case, for 3 h at room temperature under gentle movement. The complexes were isolated using Protein A-Sepharose beads, separated by 15% SDS-PAGE and the gels were exposed to radiography films (Hyperfilm-Max, Amersham) for 2 days. Identities of the viral proteins are indicated on the far right. Lane 1, antiserum H1/66; lane 2, MAb HEF; lane 3, MAb NP; lane 4, MAb M; lane 5, MAb control.

Furthermore, the variability of viral gene expression during persistence can be extended to a related RNA virus group. Measles virus expression has been conclusively shown to be regularly limited during latent conditions (Celma & Fernandez-Munoz, 1992). By comparison poliovirus persistence is subject to interfering expression phases during establishment in cell culture. Only persistent maintenance may permanently stabilize abortive and productive features of infection (Borzakian et al., 1992).

The PCR data offer evidence that active virus replication is suppressed at times. Plus strand-specific RNA detection correlates with the appearance of viral proteins, whereas minus strand RNA seems to last over long, inactive periods. These findings are supported by the observation of a long-term, non-productive persistence of Sindbis alphavirus in mouse brains, in which RNAs were detected up to 17 months after infection without viral reactivation (Levine & Griffin, 1992). Examinations of the intracellular stability of non-integrated human immunodeficiency virus genomes led to the suggestion of a quiescent state of incomplete reverse transcription products (Zack et al., 1990). In addition, mutant influenza A virus was shown to retain viral genes independently from protein synthesis beyond productive replication (Urade et al., 1993). Influenza C virus genes are irregularly expressed during persistence and viral minus strand RNA can be latently passaged in
Fig. 3. Indirect immunofluorescence test, showing levels of expression and localization of specific viral antigens. Persistently infected MDCK cells were cultured on glass slides at 32 °C. At different times after seeding, cells were fixed in ice cold acetone for 10 min and utilized for antigen detection. MAbs directed against influenza C virus HEF, NP and M proteins were used in conjunction with fluorescein isothiocyanate-coupled secondary anti-mouse IgG antibody. (a) Non-productive phase, 4 days after seeding; (b) productive phase, 8 days after seeding, (c) productive phase, 16 days after seeding.

Fig. 4. For legend see opposite.
a non-productive cell culture. Experiments to analyse the stage of activation of viral gene expression in more detail are in progress.

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References


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Fig. 4. Reverse transcription of viral RNA followed by PCR (a) and a second round of PCR, using nested primers (b). RNA amplification protocols for the influenza C virus HEF segment have been described elsewhere (Manuguerra et al., 1993). Strand specificity of the amplification was achieved by the alternative use of positive- or negative-sense primers in the RT reaction as described in the text. Ten μg of total RNA, prepared from 10^6 MDCK cells, was assayed in each case. The PCR products were separated by electrophoresis on 1% (a) and 1.5% (b) agarose gels. The sizes of the PCR products were confirmed by comparison with nucleic acid size markers (Boehringer Mannheim). Lane v is a virus control, derived from RNA prepared from egg-grown influenza C/Ann Arbor/1/50 virus persisting strain; lane m is a mock control, derived from RNA prepared from uninfected MDCK cells; lanes 1, 2, 3 and 4 contain RNA prepared from persistently infected MDCK cells 2, 6, 10 and 16 days after seeding, respectively.